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Mechanisms involved in intracellular calcium mobilization in isolated rat islets of Langerhans

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1. The rate of $^{45}Ca^{2+}$ efflux from prelabelled rat islets of Langerhans was stimulated by carbachol in a dose-dependent manner. 2. Significant stimulation occurred in the presence of 0.2 μM-carbachol; the response was half-maximal at 3–5 μM and was maximal at 20 μM. 3. Stimulation of $^{45}Ca^{2+}$ efflux by carbachol was not dependent on the presence of extracellular Ca^{2+} and was enhanced in Ca^{2+}-depleted medium. 4. Stimulation of $^{45}Ca^{2+}$ efflux by 5 μM-carbachol occurred independently of any change in [^{3}H]arachidonic acid release in prelabelled islets, and probably reflected generation of inositol trisphosphate in the cells. 5. The amphipathic peptide melittin failed to increase islet-cell $^{45}Ca^{2+}$ efflux at a concentration of 1 μg/ml, and caused only a modest increase at 10 μg/ml. 6. Despite its failure to increase $^{45}Ca^{2+}$ efflux, melittin at 1 μg/ml caused a marked enhancement of ^{3}H release from islets that had been prelabelled with [^{3}H]arachidonic acid. 7. The stimulation of ^{3}H efflux caused by melittin correlated with a dose-dependent increase in the unesterified [^{3}H]arachidonic acid content of prelabelled islets and with a corresponding decrease in the extent of labelling of islet phospholipids. 8. Combined addition of melittin (1 μg/ml) and 5 μM-carbachol to perifused islets failed to augment $^{45}Ca^{2+}$ efflux relative to that elicited by carbachol alone. 9. The data indicate that melittin promotes an increase in arachidonic acid availability in intact rat islets. They do not, however, support the proposal that this can either directly reproduce or subsequently modify the extent of intracellular Ca^{2+} mobilization induced by agents that cause an increase in inositol trisphosphate.

INTRODUCTION

Stimulation of insulin secretion from the pancreatic β-cell is believed to be initiated by a rise in the free cytosolic Ca^{2+} concentration [1]. With nutrient stimuli (e.g. glucose), much of this Ca^{2+} is derived from extracellular sources, and enters the cell through voltage-sensitive Ca^{2+} channels, which open in response to membrane depolarization [1–3]. However, islet cells also contain intracellular Ca^{2+} stores which can be mobilized in response to specific stimuli. In particular, the β-cell is equipped with muscarinic cholinergic receptors [4] and, in common with a variety of tissues, stimulation of these receptors leads to the breakdown of phosphatidylinositol bisphosphate and the generation of inositol trisphosphate [5–7]. This, in turn, induces the mobilization of stored Ca^{2+} from a membrane-bound pool [8–10].

In addition to this mechanism, it has been demonstrated, in a permeabilized islet system, that intracellular stored Ca^{2+} can also be mobilized upon incubation with arachidonic acid [11]. The inositol trisphosphate- and arachidonic acid-sensitive Ca^{2+} pools both appear to reside in the endoplasmic reticulum, although the two pools are functionally separate, since simultaneous addition of both agents to permeabilized islets elicits an additive release of Ca^{2+} [11]. Since glucose causes an increase in both inositol trisphosphate formation [5,12,13] and arachidonic acid release [11,14,15] in intact islets, the suggestion was made that these molecules may act in concert to induce mobilization of intracellular Ca^{2+} [11,13].

In the present work we have investigated this possibility by studying the effect of agents that cause increases in inositol trisphosphate and arachidonic acid on Ca^{2+} mobilization in intact rat islets of Langerhans. We have monitored the efflux of $^{45}Ca^{2+}$ from preloaded islets as an index of intracellular Ca^{2+} release and have examined the response to low concentrations of carbachol (which preferentially generates inositol trisphosphate) and melittin (which liberates arachidonic acid). The data demonstrate that carbachol induces the mobilization of intracellular Ca^{2+} in intact islets, but provide no firm evidence that this response can be modulated by an increase in arachidonic acid availability.

EXPERIMENTAL

Materials

Carbamoylcholine chloride (carbachol), arachidonic acid and melittin were obtained from Sigma. Collagenase for islet isolation was purchased from Boehringer, and bovine serum albumin (Fraction V) was from Armour Pharmaceuticals. $^{45}Ca^{2+}$ and [^{3}H]arachidonic acid were purchased from Amersham International. All other reagents were of analytical grade.

Isolation of islets of Langerhans

Islets of Langerhans were isolated from male Wistar rats (250–350 g body wt.) by collagenase digestion [16]. The isolation medium was a bicarbonate-buffered salt solution [17] containing glucose (4 mM) and $CaCl_2$

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(2 mM). This medium was equilibrated with O_2/CO_2 (19:1) and was at pH 7.4. During perifusions the incubation medium was supplemented with bovine serum albumin (1 mg/ml).

$^{45}Ca^{2+}$-efflux measurements

$^{45}Ca^{2+}$-efflux experiments were performed as described previously [18]. In brief, groups of 100 islets were preloaded with $^{45}Ca^{2+}$ (200 μCi/ml) by incubation in the presence of 20 mM-glucose for 30 min at 37 °C. The islets were then transferred to perifusion chambers and perifused at a flow rate of 1 ml/min. Experiments were commenced after a 45 min equilibration period.

Islet-cell arachidonic acid release

***(a)* Static studies.** Groups of 30 islets were incubated in 25 μl of medium which contained 1 μCi of arachidonic acid (sp. radioactivity 100 Ci/mmol) for 90 min at 37 °C. After this time, the islets were washed with 2 × 0.5 ml of fresh medium containing 4 μg of unlabelled arachidonic acid/ml, and preincubated in 50 μl of this medium for a further 10 min. A second 50 μl of medium was then added, which contained melittin at twice its final concentration. Incubation was continued for 15 min and then terminated by addition of 0.4 ml of acidified methanol (0.1 M-HCl; −20 °C). The islets were disrupted with ultrasound (70 W for 10 s), and 0.4 ml of chloroform was then added, followed by 0.3 ml of 2 M-KCl. The organic layer was removed and dried under a stream of N_2 before resuspension in 60 μl of chloroform/methanol (9:1, v/v). The extract was applied to silica-gel t.l.c. plates (Merck) which were developed in diethyl ether/benzene/ethanol/acetic acid (200:250:10:1, by vol.). Phospholipids and non-esterified fatty acids were detected with iodine vapour and scraped into scintillation vials. To each vial 0.4 ml of methanol/water/HCl (80:20:1, by vol.) was added, and the ^3H content of each fraction determined by liquid scintillation counting.

***(b)* Dynamic studies.** For measurement of the dynamics of islet-cell [^3H]arachidonic acid release, groups of 100 islets were prelabelled by incubation for 90 min at 37 °C in 40 μl of medium containing 20 mM-glucose and 2.5 μCi of [^3H]arachidonic acid (sp. radioactivity 128 Ci/mmol). The islets were then washed and transferred to perifusion chambers [18] and perifused at a flow rate of 1 ml/min. Perifusion was continued for a 30 min equilibration period, and test agents were introduced at the end of this time. Fractions were collected at 1 min intervals and samples removed for measurement of their ^3H content by liquid-scintillation counting.

RESULTS

Effects of carbachol and melittin on islet-cell $^{45}Ca^{2+}$ efflux

The data presented in Fig. 1 demonstrate that treatment of perifused rat islets with carbachol caused a dose-related increase in $^{45}Ca^{2+}$ efflux when the medium contained 4 mM-glucose. $^{45}Ca^{2+}$ efflux was measurably increased by as little as 0.2 μM-carbachol and was maximally stimulated on incubation with 20 μM-carbachol. In confirmation of previous studies [6,19,20], these effects were found to be independent of extracellular Ca^{2+}. Indeed, the efflux of $^{45}Ca^{2+}$ induced by carbachol was further increased when islets were perifused in medium containing no added Ca^{2+}. Under these conditions, the average rate of $^{45}Ca^{2+}$ efflux integrated over the 10 min after carbachol addition was increased from 186.8 ± 4.1% (in the presence of 2 mM-Ca^{2+}) to 222.7 ± 5.5% ($n = 4$ in each case; $P < 0.001$). These data suggest that the release of $^{45}Ca^{2+}$ represented true intracellular Ca^{2+} mobilization. In contrast, addition of melittin (1 μg/ml) to the perifusion medium failed to cause any change in the rate of $^{45}Ca^{2+}$ efflux (Fig. 2), despite the marked stimulation of insulin secretion that occurs under these conditions [21,22]. Increasing the melittin concentration to 10 μg/ml promoted a modest increase in $^{45}Ca^{2+}$ efflux (Fig. 2), although the extent of this release was still considerably less than that induced by a maximally effective dose of carbachol (Fig. 2). If these experiments were repeated in medium lacking added $^{40}Ca^{2+}$, melittin (1 μg/ml) also slightly augmented the rate of $^{45}Ca^{2+}$ efflux. Under these conditions, $^{45}Ca^{2+}$ efflux was increased by 29.7 ± 12.6% ($n = 4$), relative to controls, over the 10 min after melittin addition.

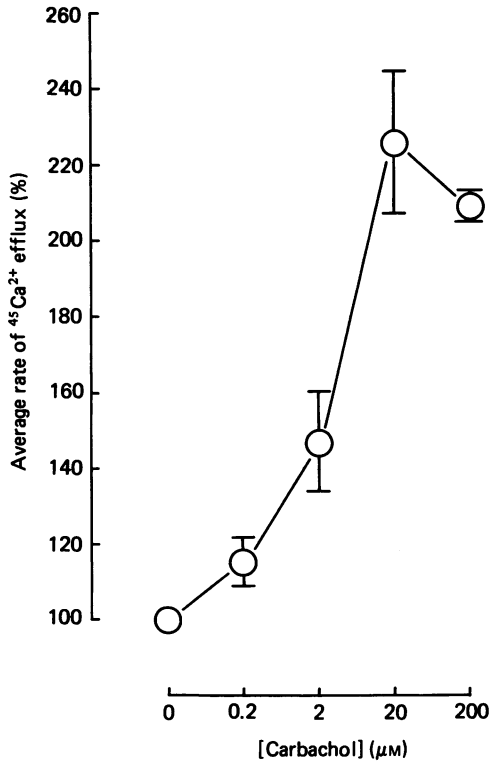

Fig. 1. Dose–response relationship for stimulation of $^{45}Ca^{2+}$ efflux by carbachol

Groups of 100 islets were preloaded with $^{45}Ca^{2+}$ and then perifused for 45 min in medium containing 4 mM-glucose. After this time carbachol was introduced and samples of the medium were collected every 1 min for measurement of their $^{45}Ca^{2+}$ content. The extent of $^{45}Ca^{2+}$ release was integrated over a 15 min period, and the results are expressed as the average rate of efflux relative to the control rate measured over the 5 min before carbachol addition (100%).

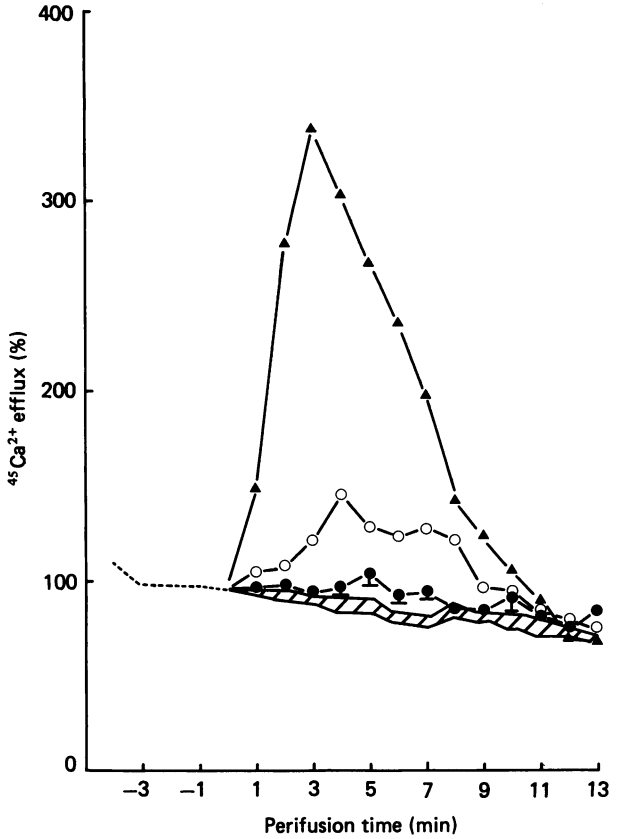

Fig. 2. Effects of melittin and carbachol on islet-cell $^{45}Ca^{2+}$ efflux

Groups of 100 islets were loaded with $^{45}Ca^{2+}$ and perifused in medium containing 4 mM-glucose. After 45 min ($t = 0$) either melittin (1 μg/ml, ●; 10 μg/ml, ○) or carbachol (1 mM; ▲) was added and the extent of $^{45}Ca^{2+}$ efflux monitored. Results are expressed as mean values for $^{45}Ca^{2+}$ efflux relative to the control rate averaged over the 5 min before introduction of the test agents (100%). The shaded area represents the control rate obtained when no addition was made. Data are from two to four perifusions for each condition.

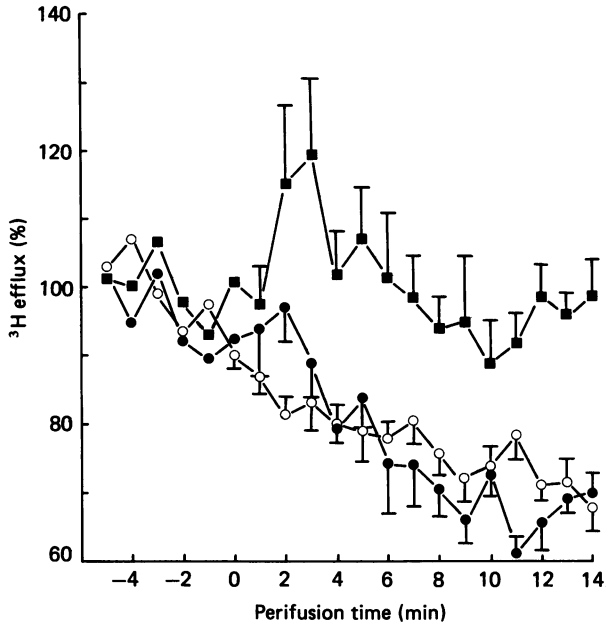

Fig. 3. Effects of melittin and carbachol on [^{3}H]arachidonic acid release from prelabelled islets

Groups of 100 islets were prelabelled with [^{3}H]arachidonic acid and then perifused for 30 min in medium containing 4 mM-glucose. After this time ($t = 0$) either melittin (1 μg/ml; ■) or carbachol (5 μM; ●) was added and the release of ^{3}H measured. Results are presented as mean values for ^{3}H efflux relative to that measured over the 5 min before introduction of the test agent (100%). The control rate obtained when no addition was made is shown by (○). Data are from four to eight perifusions for each condition.

Effects of carbachol and melittin on arachidonic acid mobilization in islet cells

Despite its inability to initiate any increase in $^{45}Ca^{2+}$ efflux, melittin (1 μg/ml) significantly stimulated the release of [^{3}H]arachidonic acid from prelabelled islets (Fig. 3). The response to melittin occurred rapidly, being evident within 2 min of addition of the agent, and was sustained throughout the 15 min period of perifusion. Over the time course of the experiment the integrated release of ^{3}H was increased from $100 \pm 4.5\%$ (control) to $129.7 \pm 5.4\%$ in the presence of melittin ($P < 0.005$). This stimulation of ^{3}H efflux by melittin was correlated with a dose-dependent increase in the radioactivity that migrated as free arachidonic acid on extraction of melittin-treated islets, and with a corresponding decrease in the label associated with islet phospholipids (Fig. 4). In contrast, carbachol, when used at a concentration that was sub-maximal for stimulation of $^{45}Ca^{2+}$ efflux (5 μM; Fig. 1), failed to augment significantly the efflux of [^{3}H]arachidonic acid from perifused islets (Fig. 3). Therefore, when used at these concentrations, carbachol appeared to mobilize $^{45}Ca^{2+}$ by a mechanism which was independent of arachidonic acid release (and presumably resulted from generation of inositol trisphosphate [6]), whereas melittin caused enhanced arachidonic acid release but no change in $^{45}Ca^{2+}$ efflux.

Effect of combined addition of carbachol and melittin on islet-cell $^{45}Ca^{2+}$ efflux

In order to study possible interactions between arachidonic acid and inositol trisphosphate in the mobilization of intracellular Ca^{2+}, islets were treated with melittin (1 μg/ml) for 5 min, and then carbachol (5 μM) was added. Under these conditions, the increase in $^{45}Ca^{2+}$ efflux elicited by carbachol was not augmented by the prior exposure to melittin (Fig. 5). This suggests that the combination of the two stimuli had not provoked any additional $^{45}Ca^{2+}$ mobilization relative to that induced by carbachol alone.

DISCUSSION

Recently much interest has centred on the role of intracellular Ca^{2+} stores as the possible source of the Ca^{2+} involved in the stimulation of the first phase of insulin secretion [1]. A role for these stores has been indicated by the observation that pancreatic islet cells are equipped with the phosphatidylinositol bisphosphate/inositol trisphosphate second-messenger system, which can be activated by both nutrients and

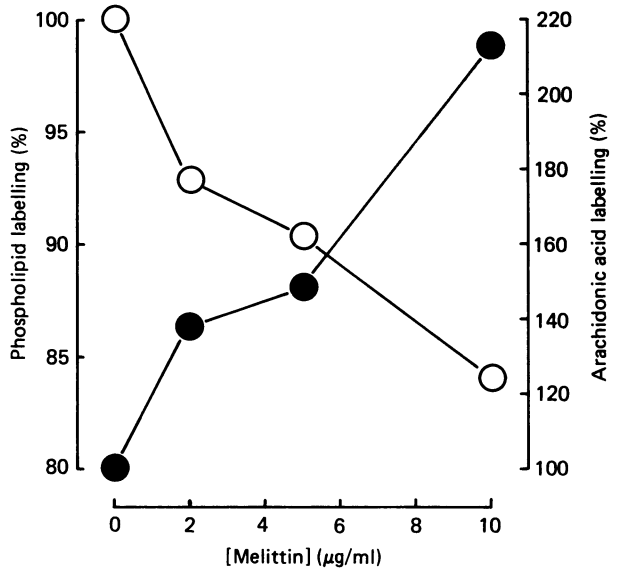

Fig. 4. Effect of melittin on [^{3}H]arachidonic acid release from islet phospholipids

Groups of islets were prelabelled with [^{3}H]arachidonic acid, then washed and incubated with melittin for 15 min. After this time the islets were treated with acidified methanol, and extracts were prepared for separation of phospholipids and non-esterified fatty acids by t.l.c. After development of the plates, the fatty acid and phospholipid spots were scraped and extracted for determination of their radioactivity. Results are mean values for the ^{3}H content of the phospholipid (○) and arachidonic acid (●) fractions, expressed relative to control islets incubated in the absence of melittin (100%). Data represent duplicate incubations from a representative experiment which was repeated three times with similar results.

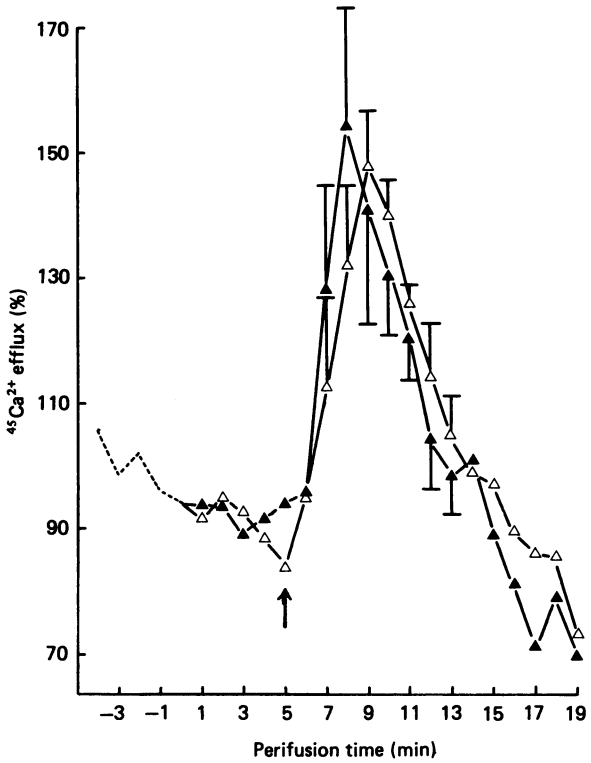

Fig. 5. Effect of combined addition of melittin and carbachol on islet-cell $^{45}Ca^{2+}$ efflux

Groups of 100 islets were preloaded with $^{45}Ca^{2+}$ and perifused for 45 min in medium containing 4 mM-glucose. After this time ($t = 0$) melittin was added to test channels (▲) and 5 min later carbachol (5 μM) was added to all channels (arrow). The rate of $^{45}Ca^{2+}$ efflux was measured and is expressed relative to the control rate averaged over the 5 min before melittin addition (100%). Data are from four perifusions in each case.

neurotransmitters [5,6]. Indeed, inositol trisphosphate causes the release of Ca^{2+} from membrane-bound stores in permeabilized islets [10] and from isolated islet-cell microsomal fractions [8,9]. It remains controversial, however, whether such Ca^{2+} release can initiate insulin secretion itself in normal islets, or whether it serves to potentiate the response to another stimulus [6,23]. In the glucose-insensitive RINm5F cell line, inositol trisphosphate-induced Ca^{2+} release does directly activate the insulin-secretory mechanism [7].

Wolf *et al.* [11] reported that a pool of Ca^{2+} located within the endoplasmic reticulum of islet cells can also be mobilized in digitonin-permeabilized islets, on treatment with arachidonic acid. Since a number of stimuli (including glucose) cause the release of arachidonic acid from islet phospholipids [11,14,15], it was suggested that arachidonic acid and inositol trisphosphate may act in a concerted manner to induce Ca^{2+} mobilization in intact islets [11,13].

We [6], and others [5], have shown previously that carbachol causes the generation of inositol trisphosphate in rat islets and elicits a large efflux of $^{45}Ca^{2+}$ from preloaded islets [6,19,20]. This response appears to reflect true mobilization of intracellular Ca^{2+}, since it is not diminished by the absence of extracellular Ca^{2+} [6,19,20]. In the present study we have confirmed these observations and demonstrate that the $^{45}Ca^{2+}$ efflux response to carbachol is dose-dependent (Fig. 1). Islet-cell $^{45}Ca^{2+}$ efflux was found to be stimulated by as little as 0.2 μM-carbachol (Fig. 1), which suggests that the response is more sensitive to carbachol than are the measurable changes in inositol lipid metabolism which are thought to cause it [24]. This apparent discrepancy probably reflects the amplification function of the inositol-lipid signalling system, but may also be related to the technical problems associated with the measurement of small changes in islet phospholipid turnover at low carbachol concentrations.

In contrast with the results obtained with carbachol, the amphipathic peptide melittin (1 μg/ml) did not alter the rate of $^{45}Ca^{2+}$ efflux from islets perifused in the presence of extracellular Ca^{2+} (Fig. 2), despite its ability to stimulate insulin secretion 4–5-fold at this concentration [21]; 1 μg of melittin/ml did, however, cause a small increase in the rate of $^{45}Ca^{2+}$ efflux from islets perifused in Ca^{2+}-depleted medium, although this response was still some 7–8-fold smaller than that elicited by carbachol under similar conditions. These data suggest that intracellular Ca^{2+} mobilization may not be a major component of the mechanism by which melittin activates the secretory mechanism of the pancreatic β-cell. In this respect, the response differs from that found in human fibroblasts, where melittin

(0.6 μg/ml) causes an increase in free cytosolic Ca^{2+} by a mechanism which is sensitive to the putative intracellular Ca^{2+} antagonist TMB-8 [25], and may therefore represent mobilization of an intracellular Ca^{2+} pool.

Despite its inability to induce an increase in $^{45}Ca^{2+}$ efflux, melittin (1 μg/ml) significantly enhanced the release of ^{3}H from islets prelabelled with [^{3}H]arachidonic acid (Fig. 3), suggesting that it had caused the liberation of arachidonic acid from membrane phospholipids. This was confirmed by the demonstration that melittin caused a dose-dependent decrease in the radioactivity associated with the phospholipid fraction obtained from islets prelabelled with [^{3}H]arachidonic acid and a concomitant increase in the ^{3}H content of the unesterified arachidonic acid fraction. These results are consistent with the suggestion that melittin causes the activation of phospholipase A_2 in islet cells [26]. Taken together, therefore, the data indicate that melittin provoked the release of arachidonic acid in islet cells, but that this did not lead to substantial mobilization of intracellular Ca^{2+}. This, in turn, implies that either the concentration of arachidonic acid in the cells was insufficient to induce large-scale Ca^{2+} release under these conditions, or that release of arachidonic acid is not a stimulus for Ca^{2+} mobilization in intact islets. It is of interest in this context that no stimulation of $^{45}Ca^{2+}$ efflux could be detected in a previous study, when intact islets were treated with exogenous arachidonic acid at concentrations up to 20 μM [11]. It was suggested that this could have been due to failure of the exogenously applied fatty acid to gain access to the cytoplasmic compartment of the β-cell. In our study this problem has been circumvented, since we have labelled the islets with [^{3}H]arachidonic acid and, in short-term incubations, labelled fatty acids are preferentially esterified to phospholipids located on the cytoplasmic face of the plasma membrane [27]. The release of ^{3}H induced by melittin is therefore likely to have reflected a direct increase in arachidonic acid availability in the cytoplasm of the islet cells. Despite this, no stimulation of $^{45}Ca^{2+}$ efflux was observed. In this context, we have previously reported that treatment of islets with 20 mM-glucose elicits only a small and slowly developing increase in $^{45}Ca^{2+}$ efflux [6], which is also inconsistent with a rapid and large mobilization of intracellular Ca^{2+}, mediated by the arachidonic acid which is released under these conditions [11]. Exogenous arachidonic acid does induce $^{45}Ca^{2+}$ release from pituitary GH_3 cells [28], although this effect has been attributed primarily to activation of Ca^{2+} extrusion, rather than to intracellular Ca^{2+} mobilization. Indeed, in these cells, the effect of arachidonic acid is to inhibit agonist-induced increases in cytosolic Ca^{2+} [29].

When the concentration of melittin was raised to 10 μg/ml, an increase in $^{45}Ca^{2+}$ efflux from perifused islets was observed (Fig. 2). At this concentration, melittin increased the arachidonic acid content of rat islets by approx. 2-fold (Fig. 4), which may indicate that large increases in free arachidonic acid can promote Ca^{2+} mobilization in islets. However, the magnitude of this response was still very modest when compared with that obtained in the presence of 1 mM-carbachol (Fig. 2). Our data, therefore, suggest that, in intact islets, the arachidonic acid-sensitive Ca^{2+} pool is, at best, very small. Furthermore, it is possible that, at high concentrations, melittin may have induced permeability changes at intracellular membrane compartments such that the observed release of Ca^{2+} occurred as a result of leakage rather than true mobilization [30,31].

In the permeabilized islet system it was demonstrated that arachidonic acid and inositol trisphosphate mobilized independent Ca^{2+} stores, since the response to each was additive when both agents were present [11]. In the present study, we have employed a dose of carbachol (5 μM) which was sub-maximal for stimulation of $^{45}Ca^{2+}$ release (Fig. 1) and which did not cause any measurable increase in arachidonic acid release from perifused islets (Fig. 3). (Higher concentrations do induce [^{3}H]arachidonic acid release in rat islets [32].) In our experiments, therefore, it is probable that the mobilization of $^{45}Ca^{2+}$ in response to 5 μM-carbachol was mediated primarily by generation of inositol trisphosphate [6]. On this basis it would be expected that any synergy between inositol trisphosphate and arachidonic acid with respect to Ca^{2+} mobilization would be revealed if arachidonic acid availability was increased in the presence of this low dose of carbachol. The data presented in Fig. 5 demonstrate, however, that no further increase in $^{45}Ca^{2+}$ efflux occurred when islets were treated with both melittin and carbachol compared with the response obtained with carbachol alone. The introduction of melittin 5 min before carbachol would have ensured that the generation of inositol trisphosphate coincided with an increase in arachidonic acid availability. These results therefore suggest that no further increase in intracellular Ca^{2+} mobilization had resulted from the presence of both agents.

In summary, therefore, our results confirm that intracellular Ca^{2+} mobilization in intact rat islets can be elicited by agents that cause an increase in inositol trisphosphate. They do not, however, support the proposal that this response can be either directly reproduced or subsequently modulated by an increase in the availability of arachidonic acid.

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